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The Acid-fastness of the Mycobacterium, especially its Correlation with the Rapidity of the Development.

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Introduction.

Observing the minute growth membranes just beginning to develop, in the previous reports⁽¹⁾, some of the questions which concerned the morphology and development process of the mycobacterium especially the tubercle bacillus were elucidated. The mycobacterium including the tubercle bacillus developed by means of the segmentation and budding. Those forms found at the base of the chain and being stained only acid-fast were considered to be nothing more than degenerative forms which had already lost the vital substance through budding, while those forms found at the top of the chain and containing the easily stainable substance or granules and at the same time being stained with the Gram and also Heidenhain methods were considered to be the viable forms of the tubercle bacillus. Furthermore, the easily stainable forms of the tubercle bacillus, namely, the mycelar, rod, and budding forms were rather easily observed, utilizing a new staining method called Ziehl-Heidenhain (author's method), within tuberculous tissues at the certain stage of the infection.

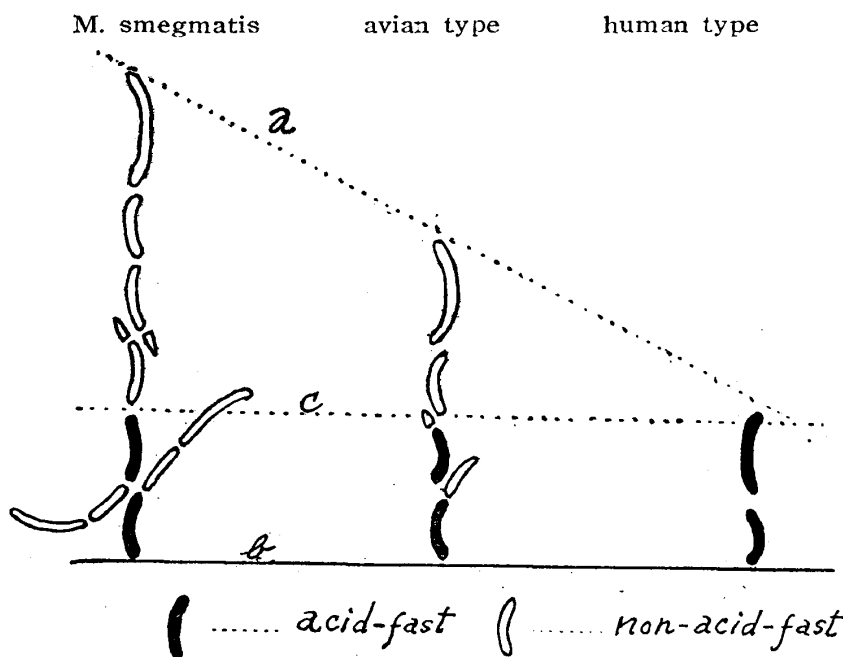
The peculiarity of the staining behavior of this kind of microorganism may be its acid-fastness. Concerning the acid-fast staining, there were those opinions of EHRlich (1886), KANAYSI (1929) etc. in the former literatures, and that of YEGIAN and KURUNG⁽²⁾ in the recent literatures. It is, however, not the subject of this paper to discuss the mechanism of this staining, but to examine the correlation between the rapidity of the development of this kind of microorganism and its acid-fast staining.

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Experiment.

1. Three types of mycobacterium. The appearance of the acid-fast substance according to the rapidity of their development.

Fig. 1. Three kinds of Mycobacterium. The rapidity of their development and the appearance of the acid-fast substance in them.



The human (strain F) and avian types (strain Chokyo) and *M. smegmatis* were employed. The procedure to make the smears was the same as that described in the previous reports. A drop of homogeneous suspension of each type of mycobacterium was added to liquid medium of Kirchner and incubated at 37°C. After one or two weeks incubation the minute growth membranes just beginning to develop were taken carefully with a platine spatule and suspended, without stirring, in a drop of saline solution placed previously on a slide. The slide was

dried in an incubator and fixed in the flame and then stained with the warm solution of Ziehl and decolorized with 3% hydrochloric acid alcohol for about ten seconds and washed in water.

The result of staining is represented in Fig. 1 as a schema. In *M. smegmatis*, the acid-fast substance began to be recognized only within the rod form which arranged at the fifth or sixth link of the chain. In the avian type, the acid-fast substance began to appear within the rod form arranging at the third or fourth link of the chain and beginning to bud. In the human type, however, the thread form found at the top of the chain was already more or less acid-fast. It seems as if Fig. 1 shows that the acid-fast substance was produced in the human type earliest of all and a little later in the avian type and latest of all in *M. smegmatis*. It may, however, become soon apparent that such a consideration as stated above was due to a misunderstanding, if one takes into consideration the difference in the rapidity of the development of these three types of mycobacterium,

Fig. 1 shows also the difference in the rapidity of the development of these three types of mycobacterium. In *M. smegmatis*, the thread form found at the top of the chain was already followed by five or six rod forms by this period of time. There were also rod forms which had already budded. In the avian type, three or four rod forms succeeded the thread form, and rod forms found at the base of the chain just began to bud. In the human type, however, the thread form just finished to produce a rod form by means of segmentation of its basal part. The difference in the rapidity of the development of these three types of mycobacterium may be shown, therefore, by the inclined line a as seen in Fig. 1, while the rapidity of the appearance of the acid-fast substance in these three types by the line c which runs parallel to the base line b.

This may indicate that the acid-fast substance appeared always with almost equal rapidity in each of these three types of mycobacterium independently of the difference in the rapidity of their development. In *M. smegmatis*, the acid-fast substance appeared only in rod forms at the base of the chain. This may not indicate the slowness of the appearance of the acid-fast substance, but the fastness of the development. In the human type, on the contrary, the thread form found at the top of the chain was already acid-fast. This may not indicate the fastness of the appearance of the acid-fast substance in this type of mycobacterium, but the slowness of its development. From these observations it may be said, in other words, that the non-acid-fast forms were easy to observe in the culture of mycobacterium developing fast and that they were difficult to find in the culture of mycobacterium which developed slowly. The following observations also supported such an understanding as stated above.

a) In *M. smegmatis* — also in the avian type — the non-acid-fast forms were easy to find in the minute growth membranes just beginning to develop on liquid medium of KIRCHNER, but it became gradually difficult to find week after week.

b) In *M. smegmatis* — also in the avian type — the non-acid-fast forms were easy to observe in the minute growth membranes just beginning to develop on liquid medium of KIRCHNER as already shown in Fig 1, but in the colonies developing in the depth of the same medium — their development was remarkably slower than the growth membranes — the thread form found at the top of the chain was already stained acid-fast just like the human type in Fig. 1.

c) *M. smegmatis* cultivated on the surface of liquid medium of KIRCHNER at 37°C was stained as seen in Fig. 1, but when one cultivated this strain on the same medium at 25°C, it was stained just like the human strain, as seen

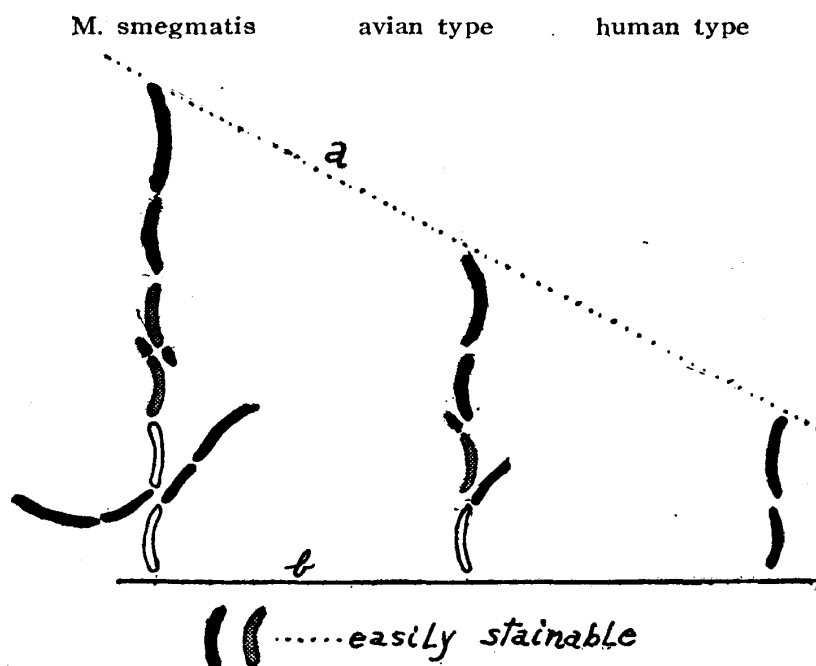
in Fig. 1.

d) It was already described in the previous report that an amount of easily stainable forms of the human strain (F) and also those of BCG was observed at the stage of their maximal development in the lymph nodes of guinea pigs inoculated subcutaneously.

From these observations, such an understanding as mentioned above is thought to be reasonable. Regardless of the type, the tubercle bacillus — mycobacterium in general — produce the acid-fast substance only within the rod form arranging at the base of the chain when its development is fast. It is, therefore, easy to find the non-acid-fast forms in such cultures. On the contrary, the acid-fast substance is produced already within the thread form found at the top of the chain, when the tubercle bacillus — mycobacterium in general — develops slowly. The non-acid-fast forms are, therefore, difficult to find in such cultures. Why was there then such difference among these three types of mycobacterium as seen in Fig. 1, in spite of the fact that they were all cultivated under the same condition? This was probably because saprophytic *M. smegmatis* was considerably promoted to develop under this condition, while the human strain was not.

2. Three types of mycobacterium. The rapidity of their development and the easily stainable substance within them.

Fig. 2 Three kinds of Mycobacterium. The rapidity of their development and the easily stainable substance in them.



The smears were made with the minute growth membranes of three types of mycobacterium in the same manner as in the above observation and stained with the alkaline solution of methylene blue of Löffler for about one minute at room temperature.

The easily stainable substance was demonstrated in these three types of mycobacterium as seen in Fig. 2. The forms bearing the easily stainable substance were called in the

previous reports the easily stainable forms and considered to be the viable

forms of the tubercle bacillus — of mycobacterium in general. Comparing Fig. 2 with Fig. 1, however, it became soon apparent that there were two kinds of forms bearing the easily stainable substance. The first forms had none of acid-fast substance but only the easily stainable substance, while the second form had more or less acid-fast substance together with the easily stainable substance.

It became also apparent from the comparison of Fig. 1 and Fig. 2 that there was another form bearing almost none of easily stainable substance but only acid-fast substance. This third form was called the acid-fast form of mycobacterium and considered to be the degenerative form which had already lost its viability after budding as discussed already in the previous reports.

3. Different results obtained through the Ziehl-Neelsen and Ziehl-Heidenhain method.

By means of the Ziehl-Neelsen method it was easy to distinguish the first form bearing only the easily stainable substance from other two forms stained acid-fast, but it was difficult to distinguish the second form bearing more or less acid-fast substance together with the easily stainable substance from the third form stained only acid-fast. The Ziehl-Heidenhain method (author's method) mentioned in the previous reports was, however, able to distinguish those forms bearing the easily stainable substance from the third form stained only acid-fast. This method could, therefore, distinguish the easily stainable forms, whether they contained some acid-fast substance at the same time or not, from the form which had only acid-fast substance, while the Ziehl-Neelsen method could only distinguish the non-acid-fast forms from other two forms containing acid-fast substance. Therefore, it may be said that the Ziehl-Heidenhain method was suitable to distinguish the viable forms of the mycobacterium from the degenerative forms.

The Ziehl-Heidenhain method (author's method)

After being stained previously with carbol-fuchsin slightly warmed for about two minutes and decolorized with 3% hydrochloric acid alcohol for about ten seconds, the minute growth membranes attached to the slide were stained as follows: Treated with 1% solution of iron alum overnight (about fifteen hours). Washed in water. Stained with 1% solution of hematoxylin (1 g of hematoxylin was dissolved in 10 cc of absolute alcohol, and then 90 cc of distilled water was added) for five hours at least. Differentiated in 2% and then 1% solution of iron alum for several minutes. Washed in water and then dried.

4. *In which manner and from what is produced the acid-fast substance?*

This may become the next question. No direct correlation was recognized between the production of the acid-fast substance and the process of budding. The easily stainable substance which was found within the thread form changed gradually into granule following the maturation and the granule became larger in the succeeding rod form. This granule was thought to be a form of the resting stage very likely to the spore of some kinds of fungi. The acid-fast substance was thought probably to have its origin in those substances which were left within the thread form or succeeding rod form and did not take part in the formation of granule and stained faintly with methylene blue of Löffler or with hematoxylin of the Heidenhain method.

Discussion.

There were many former literatures, for example, those of NOCARD and ROUX (1887), METCHNIKOFF (1888), DIXON (1889), FISCHER (1893), CRAIG (1898) etc. which had discussed the non-acid-fast mycelar, branched or granular form of the tubercle bacillus found in cultures. In those discussions, however, one did not pay much attention to the conditions under which the tubercle bacillus was promoted to develop.

From the above mentioned observations the following consideration may be possible. Not with regard to the type of the mycobacterium, the acid-fast substance is produced within the rod forms arranging at the base of the chain, when the development of the mycobacterium is promoted. It is, therefore, easy to observe the non-acid-fast forms in such cultures. On the contrary, the acid-fast substance is already produced within the thread form arranging at the top of the chain, when the mycobacterium develops slowly. It is, therefore, difficult to find the non-acid-fast forms in such cultures.

There were also several former literatures, for example, those of BAPÈS and LEVADITI (1897), FRIEDRICH (1897) etc. which referred to the non-acid-fast forms of the tubercle bacillus within living tissues. This is, above all, important for the tubercle bacillus did not always appear as the acid-fast form within tuberculous tissues as described in the previous report. In the previous report, through the Ziehl-Heidenhain method, an amount of the hematoxylin-stained mycelar and branched forms of the tubercle bacillus was observed easily in the smears of the lymph nodes of guinea pigs inoculated subcutaneously. It is to be noticed that these hematoxylin-stained forms were observed, above all, easily within the lymph nodes in which the tubercle bacillus was at the stage of his maximal development.

These findings may support not only such a consideration as mentioned above concerning the correlation between the rapidity in the development of the tubercle bacillus and the acid-fastness, but may also afford some suggestions to re-examine those opinions concerning the histogenesis, immunity etc. of tuberculosis.

Summary.

From the observations of the minute growth membranes of the tubercle bacillus and *M. smegmatis*, just beginning to develop, it may be summarized as follows:

The acid-fast substance was produced only within the rod forms arranging at the base of the chain, when the development of the mycobacterium was promoted. The non-acid-fast forms were, therefore, easy to observe in such cultures. On the contrary, the acid-fast substance was already produced in the thread form arranging at the top of the chain, when the mycobacterium developed slowly. It was, therefore, difficult to find the non-acid-fast forms in such cultures.

The non-acid-fast forms of the human strain were generally difficult to find in the culture. This may be because the human strain was not specially promoted to develop in usual culture media. It is, however, noticeable that the non-acid-fast forms of the human strain were easy to observe at the stage of their maximal development in animal tissues.

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